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Botulinum Neurotoxin type E was crystallized and an electron density map was analyzed at 3Å resolution. A new crystal form was also obtained, and may be helpful in improving the phasing. Co-crystallization/soaking experiments with gangliosides have also been undertaken. The structure of a complex between tetanus toxin C-fragment and doxorubicin has been determined, and drug binding occurs at the ganglioside binding site. Structure determination of a complex between tetanus toxin C fragment and the ganglioside GD1b is underway, and data have been collected.

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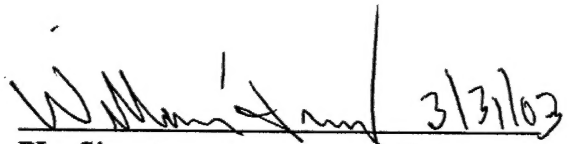

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X-ray Crystallography of *Clostridium botulinum* Neurotoxins

Work in the project covered by this report involved three different areas: (1) efforts directed at determining the crystal structure of *Clostridium botulinum* neurotoxin type E (BoNT/E); (2) efforts towards obtaining crystals of BoNT/E in complex with gangliosides or ganglioside fragments involved in binding the toxin to its receptor; (3) efforts directed at obtaining crystals of ganglioside-protein complexes with tetanus toxin C fragment. The latter was undertaken due to the structural similarity and similarity of receptor binding between the two toxins, and the fact that good diffracting tetanus toxin C fragment crystals can be readily grown. Thus these complexes may serve as useful models for BoNT/E-receptor fragment complexes.

Purified *Clostridium botulinum* neurotoxin type E (EC 3.4.24.69) precipitated with 60% ammonium sulfate was purchased from the Food Research Institute, Madison, Wisconsin, USA. It was stored at 10 °C until the toxin was prepared for crystallization. An SDS gel of the native toxin showed a single band of 150 kDa. All but about 5% of the protein was in the un-nicked form. The protein in ammonium sulfate solution was centrifuged in a refrigerated microcentrifuge for approximately five hours at 5000 rpm. The toxin was recovered by removing the supernatant and dissolving in 50 mM Hepes buffer, 100 mM NaCl at pH 7.2. This solution was then dialyzed against 50 mM Hepes buffer, 100 mM NaCl at pH 7.2 for two days to remove ammonium sulfate completely. The dialysate was changed four times during this period. The toxin was concentrated using Millipore concentrators to a final concentration of about 8 mg/ml. Our experience has shown that the above steps have to be carried out very carefully with minimal disturbance to the toxin. When the toxin was dissolved in a solution care was taken not to shake the vial but let it sit for awhile until the entire toxin goes into solution. For reasons not clear yet, this protein behaved differently from the toxin purchased from Porton Down, UK and we had to change the crystallization conditions. However, the crystals obtained were of a different morphology and were very thin plates. The diffraction quality was not very good. Microseeding produced better crystals but still the diffraction quality was poor, extending only to 3.3Å resolution initially. The crystallization conditions were improved after numerous modifications and trials and the diffraction limit enhanced to ~2.5Å.

It should be noted that frequently the protein does not yield crystals. This may be due to the fact that different preparations of protein behave differently. A picture of *Clostridium botulinum* neurotoxin type E (BoNT/E) crystals is reproduced in Figure 1.

We also tried crystallizing BoNT/E in the presence of a 40mer SNAP-25, a peptide substrate for the neurotoxin. Although no conclusions about the crystal contents can be reached at this time, crystals have been grown in the presence of the SNAP-25 40mer and diffract to 2.5Å resolution.

A native data set was collected at liquid nitrogen temperature. Crystals have the space group $P2_1$ with cell dimensions a = 81.4, b = 172.6, c = 137.3 Å and β = 99.8°. Exposure to x-radiation damages the crystals in spite of collecting data at 100°K. A native data set has been collected that is 80% complete to 2.5Å resolution with the highest shell 25% complete. However, it is 98% complete at 2.74Å resolution. The overall R_{sym} is 0.086 with a redundancy of 3.5. A diffraction pattern is shown in Figure 2.

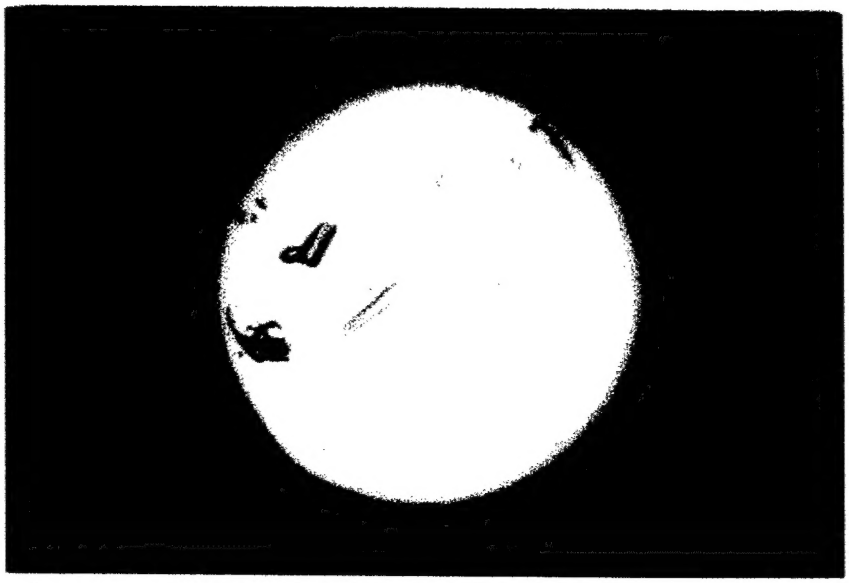


Figure 1: Crystals of *Clostridium botulinum* neurotoxin type E

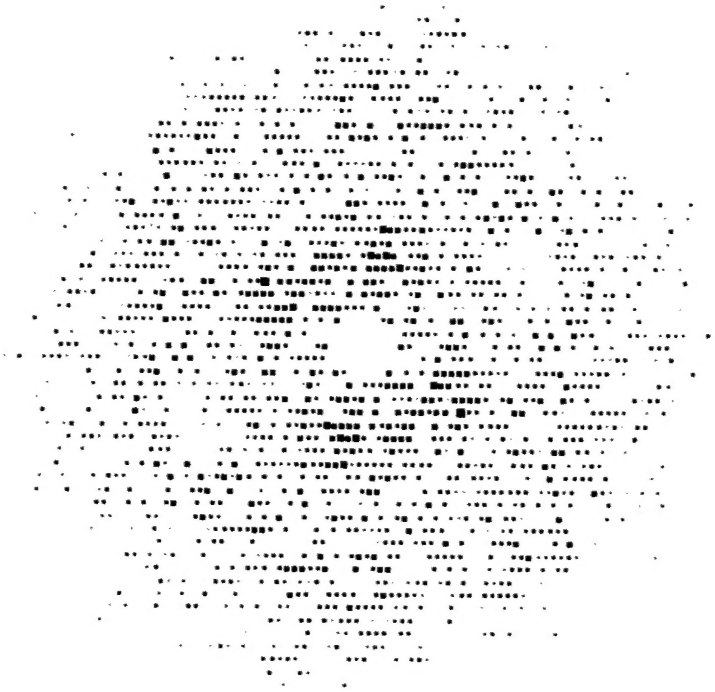


Figure 2: Pseudo precession photo of the diffraction pattern from a BoNT/E native crystal. The h0l zone is shown. Data were collected at liquid nitrogen temperature from a flash frozen crystal at the NSLS beam line X25. The edge of the pattern corresponds to 2.5Å resolution.

All *Clostridium botulinum* neurotoxins share significant sequence homology and are expected to have similar folds. However the Molecular Replacement method failed to yield an interpretable map when tried with BoNT/A or BoNT/B as a search model. In view of this a heavy atom derivative search was undertaken to determine the structure by the Multiple Isomorphous Replacement with Anomalous Signal method (MIRAS). Tungsten derivative data were collected and an electron density map was calculated, but it was not readily interpretable.

We recollected the tungsten derivative data by modifying the soaking conditions and obtained a better Patterson map as shown in Figure 4. The data extended to 3.0Å resolution and we could identify 4 heavy atom sites. This set gave better phasing statistics than the previously collected tungsten derivative data.

Derivative data were collected from BoNT/E crystals soaked in potassium hexachloroosmate IV. The data extended to 3.3Å resolution with an overall R_{merge} of 0.15. Attempts to collect MAD data at three wavelengths from the same crystal were unsuccessful since these crystals suffer radiation damage in a few hours of exposure to x-rays even though the crystals were flash frozen. The probable cause of this may be due to a combination of the nature of the crystal and the very high intensity of the x-rays. We also collected derivative data from crystals soaked in potassium hexachloroplatinate IV and potassium gold cyanide. Though they gave reasonable and interpretable Patterson maps, they did not add much to the phasing of the data.

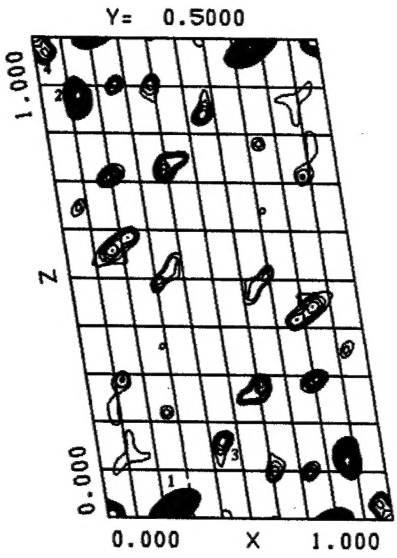


Figure 3: U,1/2,W section.

We then tried to collect MAD data on derivative crystals soaked in potassium hexabromoosmate IV to improve the phasing, but this did not work out very well. We had expected to collect MAD data at the peak, inflection and remote wavelengths of osmium and at the peak and inflection wavelengths of bromine from the same crystal. However, because of radiation damage, we could not collect MAD data from a single crystal. Data were therefore collected from separate crystals at the osmium and bromine absorption edges. Since the crystals did not diffract to a high resolution the bromine data did not add much value to the phasing. As a test case we had done this experiment on BoNT/B which gave excellent results. However, BoNT/B crystals diffracted to better than 2.0Å resolution. We attribute the failure of this method to the poor diffraction quality of BoNT/E crystals. Nevertheless, we could obtain the heavy atom positions of osmium both from a difference Patterson map and from a cross difference Fourier phased from the tungsten data. A Harker section for the osmium data is shown in Figure 4.

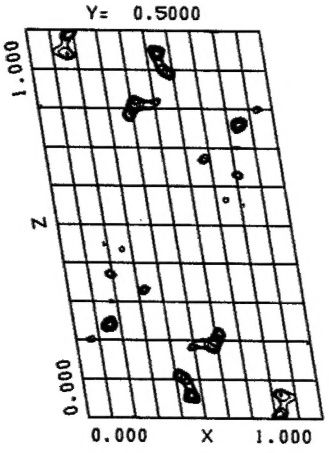
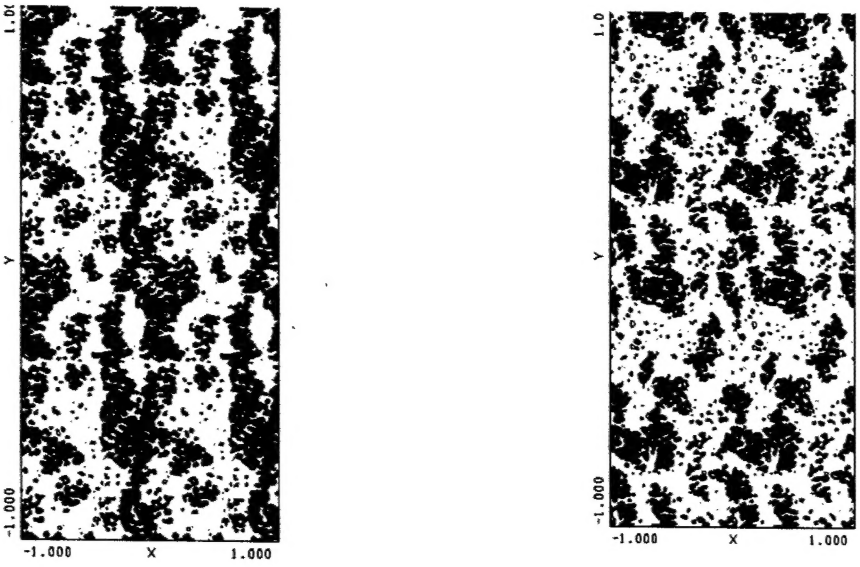


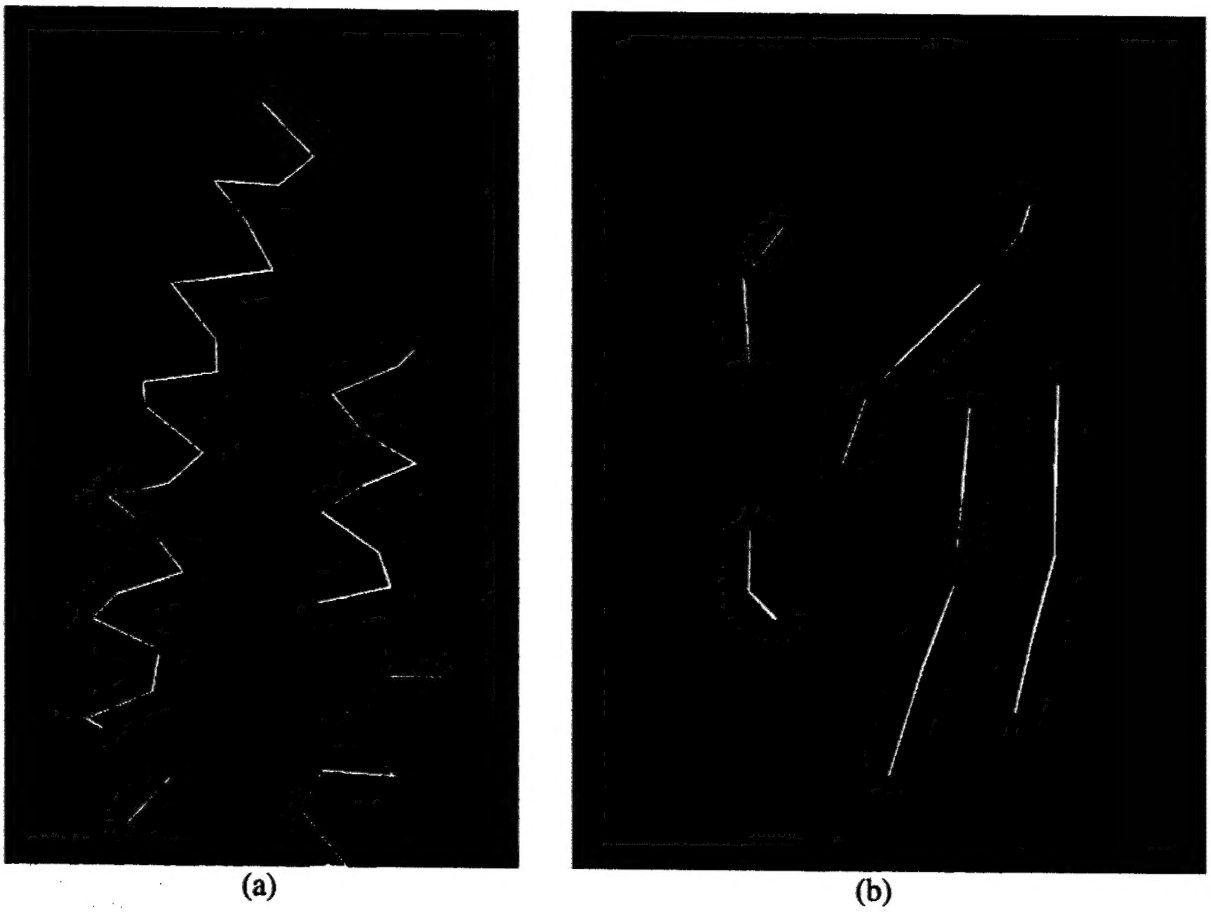
Figure 4: U,1/2,W section.

Even though we had more than two derivative data sets, we used only these two sets to calculate MIRAS phases. However, for each of these derivatives, we collected data both at the peak and inflection wavelengths. Initially the phases were calculated for data extending to 4Å resolution. The final figure of merit before solvent flattening was 0.65 and the phasing powers were in the range of 1.6 to 2.2. The phases were improved by solvent flattening/negative density truncation and non-crystallographic symmetry averaging, and were further extended slowly to 3.0Å resolution. A representative region of the MIRAS electron density map is shown below in Figure 5. The boundaries of the molecule are clearly defined and the secondary structural elements are fairly clear. We have now traced part of the polypeptide chain as shown in Figure 6. In both pictures 9Å projections are shown viewed down the c-axis. (a) shows long helical regions corresponding to the translocation domain, while (b) shows regions of β strands.



(a) (b)

Figure 5. Representative regions of the MIRAS map.



(a) (b)

Figure 6. Representative C-alpha traces superposed on the electron density map. (a) A helical region of the polypeptide chain. (b) a four stranded β-sheet.

BoNT/E new crystal form

Crystallization screening trials were continued in parallel with the other structure determination efforts in an attempt to crystallize BoNT/E in a different crystal form yielding better diffraction quality, and perhaps a more successful heavy atom derivative search. A new crystal form would also enable cross-crystal NC-symmetry averaging to be used as an additional source of phase information. Various trials with the neurotoxin purchased from the Food Research Institute, Wisconsin did not yield any new crystal forms. Recently however, we bought BoNT/E from a different vendor and have been more successful. With this sample we have succeeded in obtaining crystals in a different crystal form. Preliminary diffraction data from the new crystals were collected at the NSLS light source. The crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions, a = 149.44, b = 147.41, and c = 172.14Å. In the preliminary experiments the crystals diffracted to 3.0Å resolution. However, this may be due to two reasons: (1) Data were collected at a wavelength of 1.75Å. At this wavelength the intensity of the x-rays falls off sharply at the beamline used; (2) the cryo-condition for crystal freezing has not yet been optimized. We are confident that with another beamline or wavelength and after optimizing freezing conditions we can collect much higher resolution data. Assuming two molecules per asymmetric unit, the Matthews coefficient is 3.16 $Å^3$/Da, which translates to a 60% solvent content and is quite reasonable. Were only one molecule present per asymmetric unit the solvent content would be an unlikely 80%. Surprisingly though, self-rotation function calculations did not reveal any non-crystallographic symmetry. However, examination of a native self-Patterson function revealed a pseudo-translation present in the structure (Figure 7). This is indicated by a strong peak (40% of the origin peak) at u = 1/2, v = 0.21 and w = 0. The presence of this peak, the Matthews coefficient, and the absence of strong peaks in the self-rotation function collectively indicate that there is indeed a non-crystallographic twofold axis present, but it is parallel to the crystal "a" axis and passes through the point y = 0.145, z = 0 or 0.5.

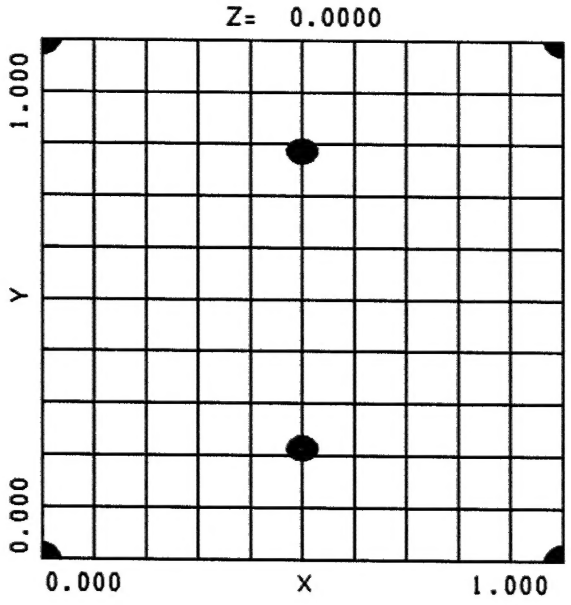


Figure 7: (uv0) section of the self-Patterson map. The pseudo translation peak is at u = 0.5, v = 0.21 and w = 0. The pseudo-translation peak is 40% of the origin peak height.

BoNT/E-Ganglioside Co-crystallization Experiments.

We have set up and are monitoring numerous crystallization experiments with the aim of obtaining a complex between BoNT/E and gangliosides or sialic acid moiety containing compounds that could bind in the toxin-receptor binding site. In particular, we set up co-crystallization experiments for BoNT/E with the gangliosides GD1a, GD1b, GT1b as well as with the sugars D-galactose, L-galactose, α-lactose, β-lactose and sialic acid. The ligand/protein concentration ratio has been varied as well as the pH, buffer, precipitant nature and precipitant concentration during the screens. Complexes of this sort should enable us to probe specific interactions in the toxin's receptor binding site. We used protein obtained from Metabiologics, Inc. as well as from Sigma Chemicals. We have been observing granular and microcrystalline precipitates in some of the setups, but no usable crystals have appeared.

Tetanus toxin C-fragment complexes with ligands

Antagonists for botulinum and tetanus neurotoxins could act in three ways: (1) they could be molecules that attach to the binding site thereby inhibiting binding of neurotoxins to gangliosides; (2) they may act before internalization to prevent internalization; or (3) they could be inhibitors which would stop the catalytic action by blocking the active site or by chelating the active site zinc.

Biochemical, electrospray ionization mass spectroscopy (ESI-MS) studies have shown that doxorubicin, a well- known DNA intercalator, binds to these neurotoxins. Determination of the crystal structure of the C-fragment of tetanus toxin complexed with doxorubicin was undertaken to identify the binding site for the drug and its interaction with the protein molecule. Crystals of the complex were prepared by soaking native rTTc crystals in the mother liquor containing doxorubicin. X-ray diffraction data were collected at liquid nitrogen temperature at the National Synchrotron Light Source, Brookhaven National Laboratory. The drug molecule was identified from a difference Fourier map and the structure was refined using the program CNS until convergence. Doxorubicin binds in a site that has previously been identified as the binding site for gangliosides. The drug stacks against the conserved residue Trp 1289 and interacts with another conserved residue, His 1271 (Figure 8).

Figure 8: Ribbons representation of the C-fragment of tetanus neurotoxin with doxorubicin (red) and the protein residues interacting with it (blue).

Tetanus Toxin C fragment (rTTc)-Ganglioside Co-crystallization Experiments.

We have attempted preparation of toxin-ligand complexes with the tetanus toxin C fragment. Co-crystallization of the complexes was attempted in the presence of the gangliosides GT1b, GD1b, GM1 and GM3 as well as with sialic acid, and the sugars α-lactose, β-lactose, D-galactose and L-galactose. Over two hundred conditions for preparing the complexes and for crystallization were tried, entailing a wide range of variation of the relative and absolute concentrations of the toxin and the ligand, times of soak, pH and temperature. The only co-crystallization setups that yielded crystals were one setup each of rTTc-GT1b and rTTc sialic acid and two setups of rTTc-GM3. All of these crystals appeared after 6 to 8 months and did not grow beyond 0.1 to 0.2 mm. Photographs of some of these crystals are shown in Figure 9 below.

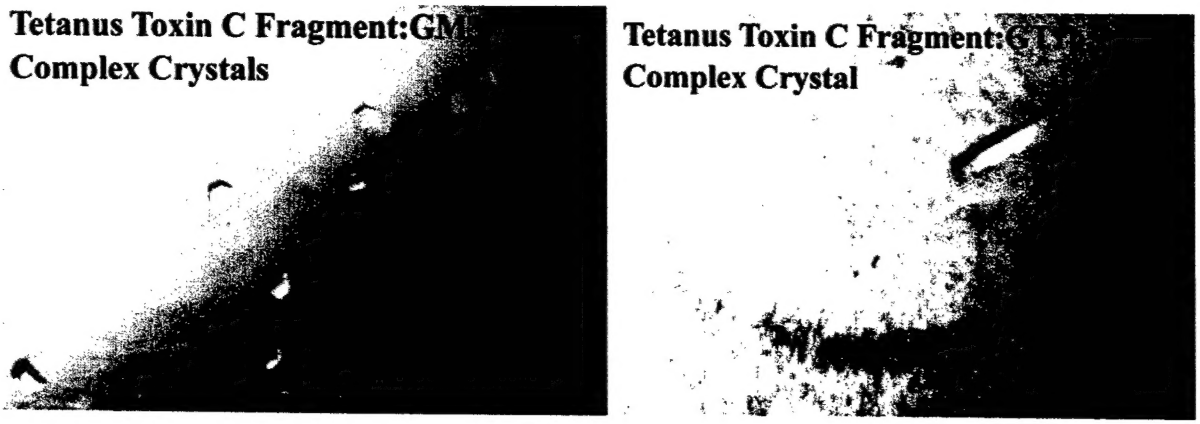


Figure 9. Tetanus toxin C fragment crystals grown in the presence of gangliosides

Unfortunately diffraction from the co-crystals shown above was very poor (~10Å resolution). It was later found that these crystals could not be grown reproducibly and subsequent attempts have failed to yield additional crystals thus far. The initial rTTc-sialic acid co-crystals diffracted to 3.6Å and a data set was collected. However, the crystal appeared to decay severely after a day's exposure to x-radiation even under cryo conditions (-180°C).

In addition to the co-crystallization experiments, we have also grown a large number of native rTTc crystals in order to attempt infusion of gangliosides and sugars into them by soaking. The mother liquor best suited for cryo-protection of the crystals has been found by extended trials. Native rTTc crystals were deposited one at a time in a 20μl drop of the mother liquor, varying concentrations of ganglioside GD1b added to the drop and the crystals soaked for different lengths of time. The concentrations of GD1b in the drops ranged from 1 to 5 mg/ml and the soaking times of the crystals from 1 to 14 days. While some of the crystals disintegrated, one of the crystals, soaked for three days in 3mg/ml GD1b, diffracted to 3.6Å and a data set was collected. Another larger crystal, soaked for 2 days in 3mg/ml GD1b, diffracted strongly to 2.3Å and a complete data set has been collected (unit cell parameters a=66.8, b=79.4, c=93.8Å, space group $P2_12_12_1$). The analysis of the data to identify the precise binding features of the ganglioside is however complicated by the fact that the structure seems to show deviations from both forms of the native rTTc structures that have been reported (Form 1: a=67.4, b=79.7, c=91.1Å, space group $P2_12_12_1$, Umland et al.1997; Form 2: a=71.2, b=79.4, c=93.8Å, space group $P2_12_12_1$, Knapp et al. 1998). While these two structures are generally conformationally similar, they show

tandem deviations of over 1Å between Cα positions in several regions. Therefore we omitted three regions of 10 to 15 residues each where the differences are most pronounced and positioned our structure in its unit cell through molecular replacement using the model co-ordinates of Knapp et al., since that structure had been determined at very high resolution (1.61Å). This model was then refined by rigid body methods and simulated annealing to R=0.295, (R_{free}=0.39). Difference maps with coefficients ($2F_O$-F_C) and (F_O-F_C) indicated electron density at greater than 2σ in several regions including those that were omitted and others that could be areas associated with ganglioside binding. Some of the omitted regions were carefully refit and subsequent refinement reduced R to 0.27 (R_{free}=0.36).

We are interpreting our data with sequential omit maps and refitting as well as 'shaking' the structure by adding a small random number between –0.3 and +0.3 to all of the coordinates before calculating electron density maps (as a method suggested by McRee to remove any model bias that had been introduced). The protein chain itself is being carefully retraced wherever necessary so that the ganglioside, if present, can be identified unambiguously and not be confused with density associated with shifted protein segments.

Reconciliation with Statement of Work.

1. The major goal of this proposal was to determine the three dimensional crystal structure of botulinum neurotoxin E (BoNT/E). We have obtained crystals of BoNT/E in two different forms and traced much of the structure for one of them based on the MIRAS electron density map. Completion of the structure is in progress. It should be mentioned that while Dr. Eric Johnson was stated as our collaborator, it was not always possible to obtain crystals from samples obtained from him. Thus, other vendors were also tried, with better success.

2. The time and effort spent on improving BoNT/E crystals was so exhaustive that we were unable to proceed with plans for crystallizing the light chain alone or the C fragment of BoNT/E. Instead, we tried obtaining complexes of gangliosides and other sugar containing compounds with the closely related C fragment of tetanus toxin (rTTc). This experiment is of much value since there exists a high sequence homology between the two toxins and a close structural similarity is expected between them, especially with regard to receptor binding. We have determined that the drug doxorubicin binds to rTTc in a site that has been previously identified through biochemical studies as the binding site for gangliosides. We have collected high resolution x-ray data for one complex of rTTc with the ganglioside GD1b and are interpreting the electron density maps.

Completion of the work.

For the native BoNT/E structure we plan to continue chain tracing and model building, while at the same time trying to improve the data resolution and phasing. For the ganglioside-BoNT/E experiments we will continue our crystallization screening efforts. For the tetanus toxin C fragment complexes, we are actively continuing analysis of the complex with ganglioside GD1b. We are also examining other data already collected, while continuing experiments to improve the resolution. As crystals of rTTc are readily obtainable, we will try even more conditions with the ligands at hand, and extend the study to include the ligands T-antigen, cellotetraose, stearic acid and different forms of polysaccharides containing the sialic acid moiety.